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CD154-Dependent Priming of Diabetogenic CD4$^+$ T Cells Dissociated from Activation of Antigen-Presenting Cells

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Summary

We followed the fate of K^d- or I-A^{g7}-restricted β cell-autoreactive T cells in monoclonal TCR-transgenic NOD mice expressing or lacking CD154. 8.3-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ mice, which bear autoreactive CD8$^+$ T cells, developed diabetes with the same incidence and tempo as 8.3-NOD.*RAG-2*$^{-/-}$/*CD154*$^+$ mice. Recruitment of *CD154*$^{-/-}$ 8.3-CD8$^+$ CTL was accelerated by *CD154*$^+$CD4$^+$ T cells, by expression of a B7.1 transgene in β cells or by treatment of the mice with CpG-DNA or an agonistic anti-CD40 antibody. In contrast, the autoreactive CD4$^+$ T cells maturing in 4.1-NOD.*RAG-2*$^{-/-}$ mice lost their diabetogenic potential if they lacked *CD154*, even in the presence of *CD154*$^+$CD4$^+$ T cells, B7.1 molecules on β cells, CpG-DNA treatment, or systemic CD40 ligation. These results demonstrate the existence of a novel, CD154-dependent pathway of CD4$^+$ T cell activation that is independent of CD40-mediated activation of APCs.

Introduction

The interaction between CD40 on professional APCs (B cells, dendritic cells, or macrophages) and CD154 (CD40 ligand) on CD4$^+$ T helper cells plays a critical role in the activation and differentiation of precytotoxic T lymphocyte precursors (Stout and Suttles, 1996; Grewal and Flavell, 1998). Ligation of CD40 on dendritic cells (DCs), for example, upregulates the expression of costimulatory molecules, increases DC survival, and elicits the production of proinflammatory cytokines by these cells (Caux et al., 1994; Cella et al., 1996; den Haan and Bevan, 2000; Grewal and Flavell, 1998; Stout and Suttles, 1996). These changes endow antigen-loaded APCs with the ability to costimulate antigen-specific CD8$^+$ and CD4$^+$ T cell responses and to foster the T cells' differentiation into cytotoxic T lymphocytes (CTL) (van Essen et al., 1995; Grewal et al., 1995, 1996; Yang and Wilson, 1996; Ridge et al., 1998; Schoenberger et al., 1998; Bennett et al., 1998). Since CD154/CD40 interactions are critical for T-T collaboration and APC-driven T cell differentiation, CD154-deficient mice cannot mount primary CD4$^+$ T cell responses to exogenous antigens (Grewal et al., 1996) or CD8$^+$ CTL responses against viruses (van Essen et al., 1995; Yang and Wilson, 1996). Although T helper-dependent CD8$^+$ T cell responses require the presence of CD154 molecules on CD4$^+$ T cells, the contribution of CD8$^+$ T cells' CD154 molecules to their own activation and differentiation is poorly understood (Roy et al., 1993; Hermann et al., 1995; Sad et al., 1997; Lefrancois et al., 1999).

Notwithstanding the fact that most of the biological effects of CD154/CD40 interactions can be accounted for by the activation of APCs by CD40 ligation, CD154 has the capacity to transduce intracellular signals and elicit T cell responses. CD40 cDNA-transfected tumor cells and agonistic anti-CD154 mAbs, for example, can trigger proliferation, cytokine production, and/or apoptosis in resting human T cells (Cayabyab et al., 1994; Peng et al., 1996; Blair et al., 2000). In addition, ligation of CD154 in Jurkat cells can induce c-Jun NH2 terminal kinase (JNK) and p38 mitogen-activated protein (MAP) kinase (Brenner et al., 1997; Koppenhoefer et al., 1997). Despite these observations, it has been difficult to dissociate the effects of CD40-induced activation of APCs on T cell responses from those mediated by CD154-transduced T cell signaling. As a result, the true biological significance of CD154 triggering in vivo remains unclear.

Development of spontaneous autoimmune diabetes in nonobese diabetic (NOD) mice is the result of a complex CD4$^+$ and CD8$^+$ T cell-dependent autoimmune process against the pancreatic β cells (Delovitch and Singh, 1997). β cell-reactive CD8$^+$ T cells are invariably present in the islets of prediabetic NOD mice (Nagata et al., 1994; Santamaria et al., 1995; DiLorenzo et al., 1998; Wong et al., 1999) and play critical roles both in the initiation of insulitis and in its progression toward overt clinical disease (Katz et al., 1993; Serreze et al., 1994; Wicker et al., 1994; Verdaguer et al., 1996, 1997; Wang et al., 1996; Amrani et al., 2000a). The recruitment, activation, and differentiation of diabetogenic CD8$^+$ T cells, however, require the assistance of CD4$^+$ T cells. Thus, splenic CD8$^+$ T cells from diabetic NOD mice cannot accumulate in pancreatic islets of irradiated NOD mice or NOD.*scid* mice in the absence of CD4$^+$ T cells (Thivolet et al., 1991; Christianson et al., 1993). Furthermore, β cells do not express costimulatory molecules and hence cannot directly drive the differentiation of naive β cell-reactive CD8$^+$ T cells into CTL (Stephens and Kay, 1995). In addition, genetic susceptibility and resistance to insulitis and diabetes are profoundly affected by polymorphisms of MHC class II genes (Tisch and McDevitt, 1996), which control the development and function of CD4$^+$ but not CD8$^+$ T cells.

CD40/CD154 blockade in young NOD mice completely prevents the onset of insulitis (Balasa et al., 1997; Green et al., 2000), suggesting that CD40/CD154 interactions play a critical role in the development, recruit-

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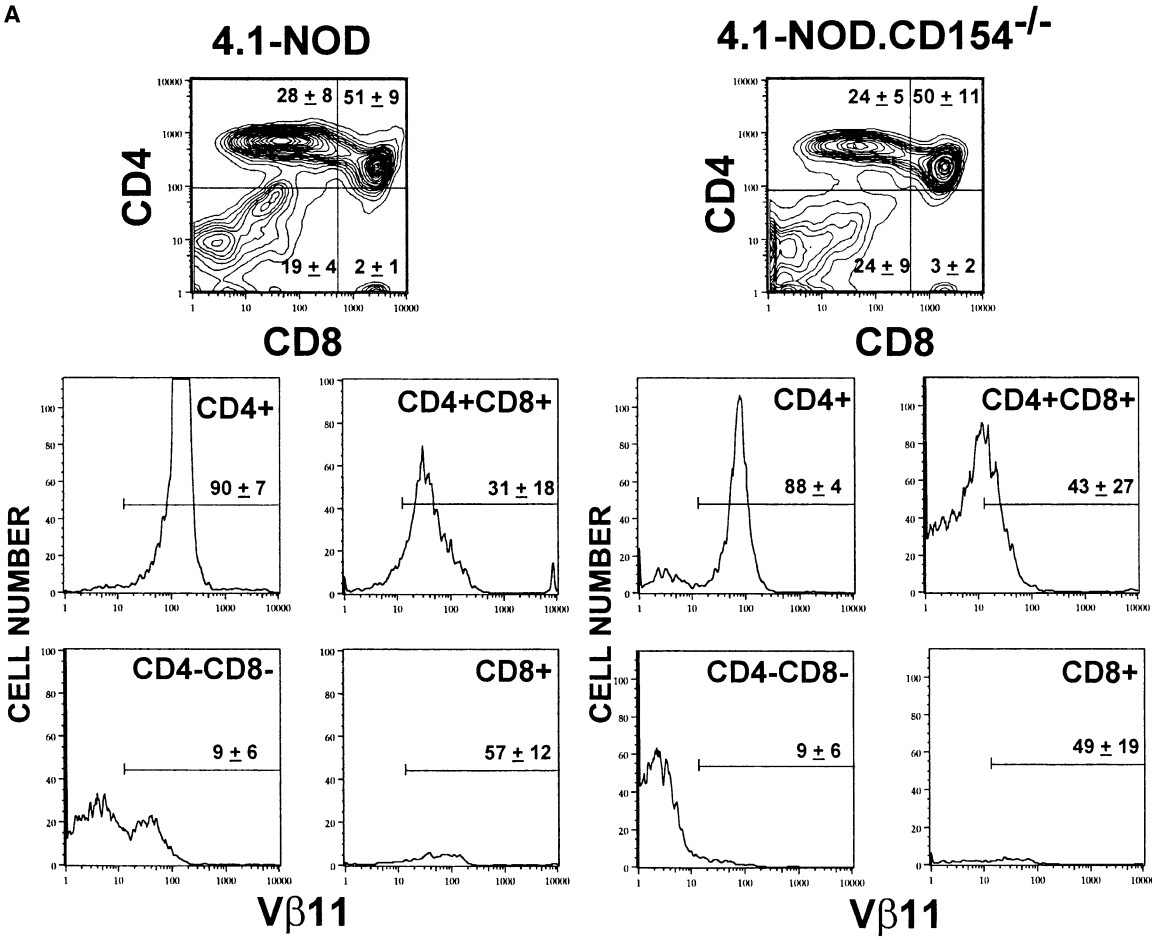
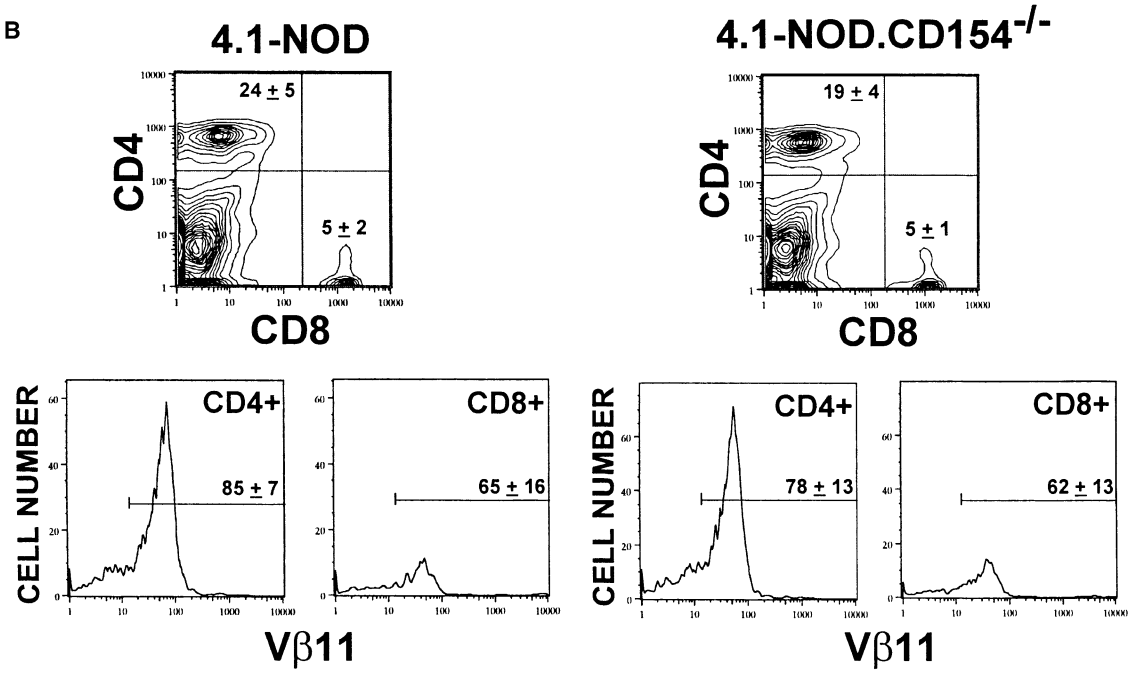

A
4.1-NOD
4.1-NOD.CD154-/-
CD4
CD8
28 ± 8
51 ± 9
19 ± 4
2 ± 1
24 ± 5
50 ± 11
24 ± 9
3 ± 2
CD4+
90 ± 7
CD4+CD8+
31 ± 18
CD4-CD8-
9 ± 6
CD8+
57 ± 12
88 ± 4
43 ± 27
9 ± 6
49 ± 19
CELL NUMBER
Vβ11


B
4.1-NOD
4.1-NOD.CD154-/-
CD4
CD8
24 ± 5
5 ± 2
19 ± 4
5 ± 1
CD4+
85 ± 7
CD8+
65 ± 16
78 ± 13
62 ± 13
CELL NUMBER
Vβ11

ment, and/or activation of diabetogenic CD4+ and/or CD8+ T cells. Here we have investigated the role of CD154 in the recruitment, activation, and differentiation of two NOD islet-derived, highly diabetogenic, MHC class I- or class II-restricted β cell-reactive T cell specificities (8.3-CD8+ and 4.1-CD4+, respectively) in TCR-transgenic, recombination-activating gene (RAG)-2-deficient NOD mice. We show that unlike 8.3-CD8+ T cells, which do not require CD154 on their surface to proliferate in pancreatic lymph nodes, to spontaneously differentiate into CTL in vivo, and to trigger diabetes, 4.1-CD4+ T cells cannot respond to β cell antigen in vivo in the absence of CD154. Constitutive expression of costimulatory molecules on β cells and activation of professional APCs in vivo with *CD154*+ CD4+ T helper cells, CpG DNA, or agonistic anti-CD40 mAb potentiates the diabetogenic potential of *CD154*-deficient 8.3-CD8+ T cells but has no effect on the diabetogenicity of *CD154*-deficient 4.1-CD4+ T cells. These data indicate that CD154 triggering is indispensable for the activation of naive autoreactive CD4+ T cells in spontaneous autoimmune diabetes and thus demonstrate the existence of a novel, CD154-dependent pathway of CD4+ T cell activation in vivo that is independent of CD40-mediated activation of APCs.

Results

CD154-Deficient 4.1-CD4+ T Cells Respond to Antigen Stimulation In Vitro but Cannot Efficiently Trigger Diabetes In Vivo

To investigate whether CD40/CD154 interactions are necessary for the development and/or activation of diabetogenic CD4+ T cells, we compared the fate of a highly diabetogenic CD4+ T cell specificity in *CD154*-competent versus *CD154*-deficient NOD mice expressing TCRαβ transgenes derived from an islet-derived CD4+ T cell clone (4.1-NOD and 4.1-NOD.*CD154*$^{-/-}$ mice, respectively) (Schmidt et al., 1997). Expression of the 4.1-TCR in 4.1-NOD mice skews thymocyte development toward the CD4+ T cell subset and results in a dramatic acceleration of the onset of diabetes, owing to massive recruitment of β cell-cytotoxic CD4+ T cells into islets (Schmidt et al., 1997; Amrani et al., 2000b). 4.1-NOD.*CD154*$^{-/-}$ mice displayed thymic, splenic, and lymph node cytofluorometric profiles that were comparable to those seen in 4.1-NOD mice (Figure 1) and exported β cell-reactive CD4+ T cells to the periphery (Figure 2A). Although the mean fluorescence intensity for Vβ11 on *CD154*$^{-/-}$ thymocytes was slightly lower than on *CD154*+ thymocytes, the differences did not reach statistical significance (99 ± 40 versus 115 ± 33 for CD4+CD8+ cells; 92 ± 5 versus 104 ± 16 for CD4+CD8− cells). The peripheral CD4+ T cells of both types of mice also expressed comparable levels of Vβ11 (mfi values: 60 ± 2 for *CD154*$^{-/-}$ cells; 65 ± 6 for *CD154*+ cells). When compared to 4.1-NOD mice, however, 4.1-NOD.*CD154*$^{-/-}$ mice developed a significantly reduced incidence (and delayed onset) of both diabetes (Figure 2B) and insulitis (Figure 2C). The few T cells that were present in islets of 4.1-NOD.*CD154*$^{-/-}$ mice produced Th1 cytokines upon stimulation with phorbol-myristate acetate and ionomycin (Figure 2D). This suggested that the *CD154* deficiency impaired the diabetogenic activity of 4.1-CD4+ T cells by interfering with their recruitment, rather than by inducing their immune deviation. This inefficient recruitment of 4.1-CD4+ Th1 cells in 4.1-NOD.*CD154*$^{-/-}$ mice was not due to absence of autoantigen-loaded APCs inside islets, as purified 4.1-CD4+ T cells proliferated efficiently in response to NOD.*CD154*$^{-/-}$ islet cells in the absence of exogenous APCs (Figure 2E). Thus, in the absence of *CD154*, 4.1-CD4+ T cells can respond to antigen in vitro but do not efficiently accumulate in pancreatic islets in vivo.

Complete Absence of Insulitis and Diabetes in Monoclonal *CD154*-Deficient 4.1-NOD Mice

To confirm that the diabetes resistance of 4.1-NOD.*CD154*$^{-/-}$ mice was due to poor recruitment of 4.1-CD4+ T cells as opposed to immune regulation by nontransgenic T cells, we compared the natural history of diabetes in 4.1-NOD.*RAG-2*$^{-/-}$/*CD154*+ versus 4.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ mice, which cannot rearrange endogenous TCR genes (Verdaguer et al., 1997). 4.1-NOD.*RAG-2*$^{-/-}$/*CD154*+ mice developed diabetes with an incidence and tempo similar to 4.1-NOD mice, but none of 47 4.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ mice developed diabetes (Figure 3A). Histopathological studies confirmed that, unlike their *CD154*+ counterparts, 4.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ mice were insulitis free (Figure 3B). Thus, CD154 is indispensable for the recruitment and/or accumulation of 4.1-CD4+ T cells into islets.

Diabetogenic CD8+ T Cells Do Not Need to Express CD154 to Trigger Autoimmune Diabetes

Development of autoimmune diabetes in wild-type NOD mice is CD8+ T cell dependent (Katz et al., 1993; Serreze et al., 1994; Wicker et al., 1994; Wang et al., 1996; Verdaguer et al., 1996, 1997; Amrani et al., 2000a). To ascertain whether diabetogenic CD8+ T cells also need to express CD154 to differentiate into CTL in vivo, we compared the fate of a diabetogenic, H-2K^{d}-restricted β cell-reactive TCR (8.3) in *CD154*-competent versus *CD154*-deficient 8.3-TCR-transgenic NOD.*RAG-2*$^{-/-}$ mice. The 8.3-TCR recognizes the peptide NRP-A7 and uses a TCRα chain that is identical to those used by a significant fraction of autoreactive CD8+ T cells recruited to islets during the earliest stages of insulitis (Anderson et al., 1999; DiLorenzo et al., 1998; Amrani et al., 2000a). We have shown that naive 8.3-CD8+ T cells are significantly more diabetogenic in the presence of CD4+ T cells than in their absence. However, the requirement for CD4+ T

Figure 1. Development of *CD154*+ and *CD154*$^{-/-}$ 4.1-CD4+ T Cells

CD4, CD8, and Vβ11 profiles of thymocytes (A) and splenocytes (B) from 4.1-NOD (n = 12) and 4.1-NOD.*CD154*$^{-/-}$ mice (n = 9). Cells were stained with anti-CD8-PE, anti-Vβ11-FITC, and anti-CD4-biotin plus Streptavidin-PerCP. Numerical values correspond to percentage ± SD of cells within each gate. None of the differences were statistically significant.

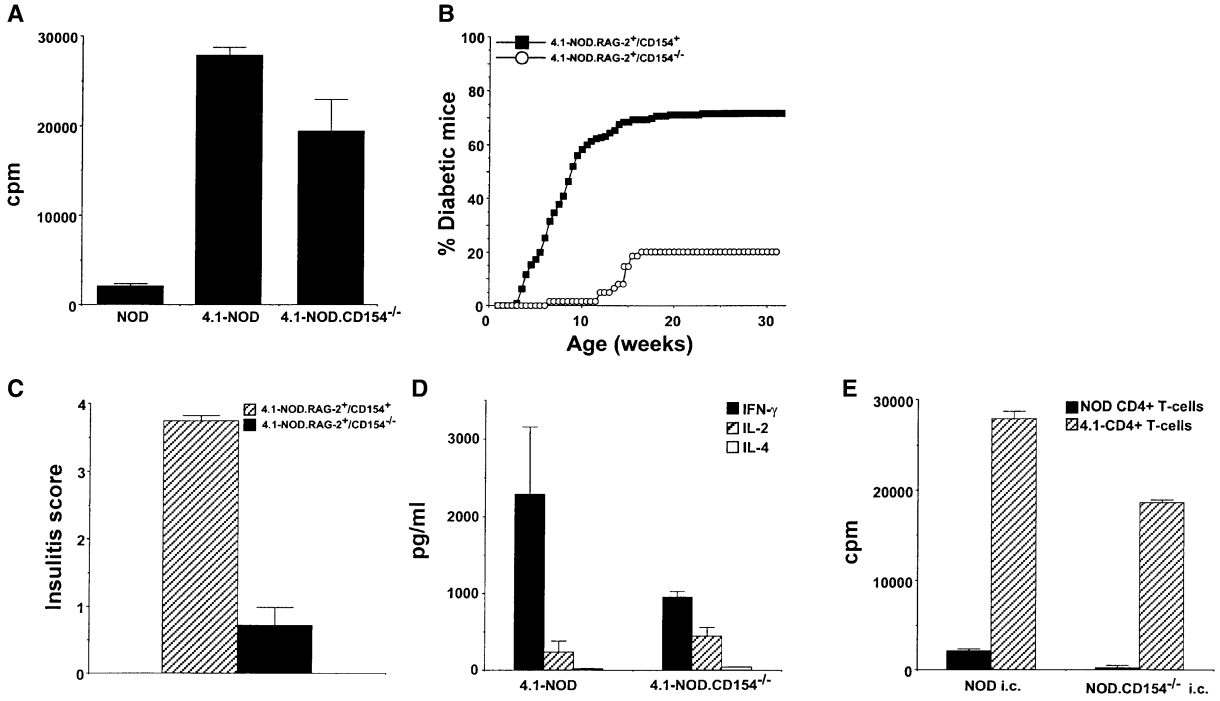


Figure 2. Function of *CD154*$^{+}$ and *CD154*$^{-/-}$ 4.1-CD4^{+} T Cells In Vitro and In Vivo

(A) Proliferation of naive splenic 4.1-CD4^{+} T cells from 4.1-NOD and 4.1-NOD.*CD154*$^{-/-}$ mice against irradiated NOD islet cells.

(B) Natural history of diabetes in female 4.1-NOD (n = 225) and 4.1-NOD.*CD154*$^{-/-}$ mice (n = 62).

(C) Insulitis scores in >20-week-old 4.1-NOD (n = 6) versus 4.1-NOD.*CD154*$^{-/-}$ mice (n = 7).

(D) Cytokine profile of islet-associated T cells in >20-week-old 4.1-NOD (n = 4) versus 4.1-NOD.*CD154*$^{-/-}$ mice (n = 6).

(E) Proliferation activity of splenic 4.1-CD4^{+} T cells from 4.1-NOD against NOD and NOD.*CD154*$^{-/-}$ islet cells.

cells is not absolute and, as a result, over 40% of 8.3-NOD.*RAG-2*$^{-/-}$ mice spontaneously develop diabetes (Verdaguer et al., 1997). 8.3-NOD.*RAG-2*$^{-/-}$ and 8.3-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ mice displayed similar thymocyte and splenocyte cytofluorometric profiles (Figure 4). The splenic CD8^{+} T cells of both types of mice proliferated equally well (Figure 5A), secreted similar levels of cytokines (IFN-γ and IL-2) (Figure 5B), and differentiated efficiently into CTL in response to NRP-A7 peptide-pulsed bone marrow-derived DCs (Figures 5C and 5D). In fact, CTL from both types of mice killed both Fas-expressing (Figure 5C) and Fas-deficient fibroblasts

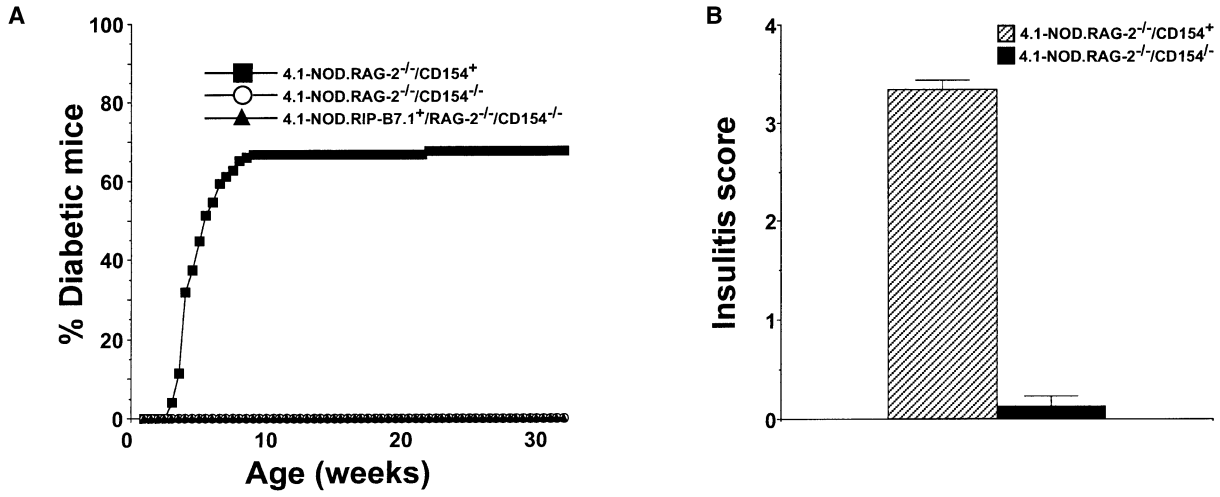


Figure 3. Diabetogenic Activity of *CD154*$^{+}$ and *CD154*$^{-/-}$ 4.1-CD4^{+} T Cells in Monoclonal T Cell NOD Mice

(A) Natural history of diabetes in female 4.1-NOD.*RAG-2*$^{-/-}$ (n = 123), 4.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ mice (n = 47), and 4.1/RIP-B7.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ mice (n = 34).

(B) Insulitis scores in >20-week-old 4.1-NOD.*RAG-2*$^{-/-}$ (n = 2) and 4.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ mice (n = 3).

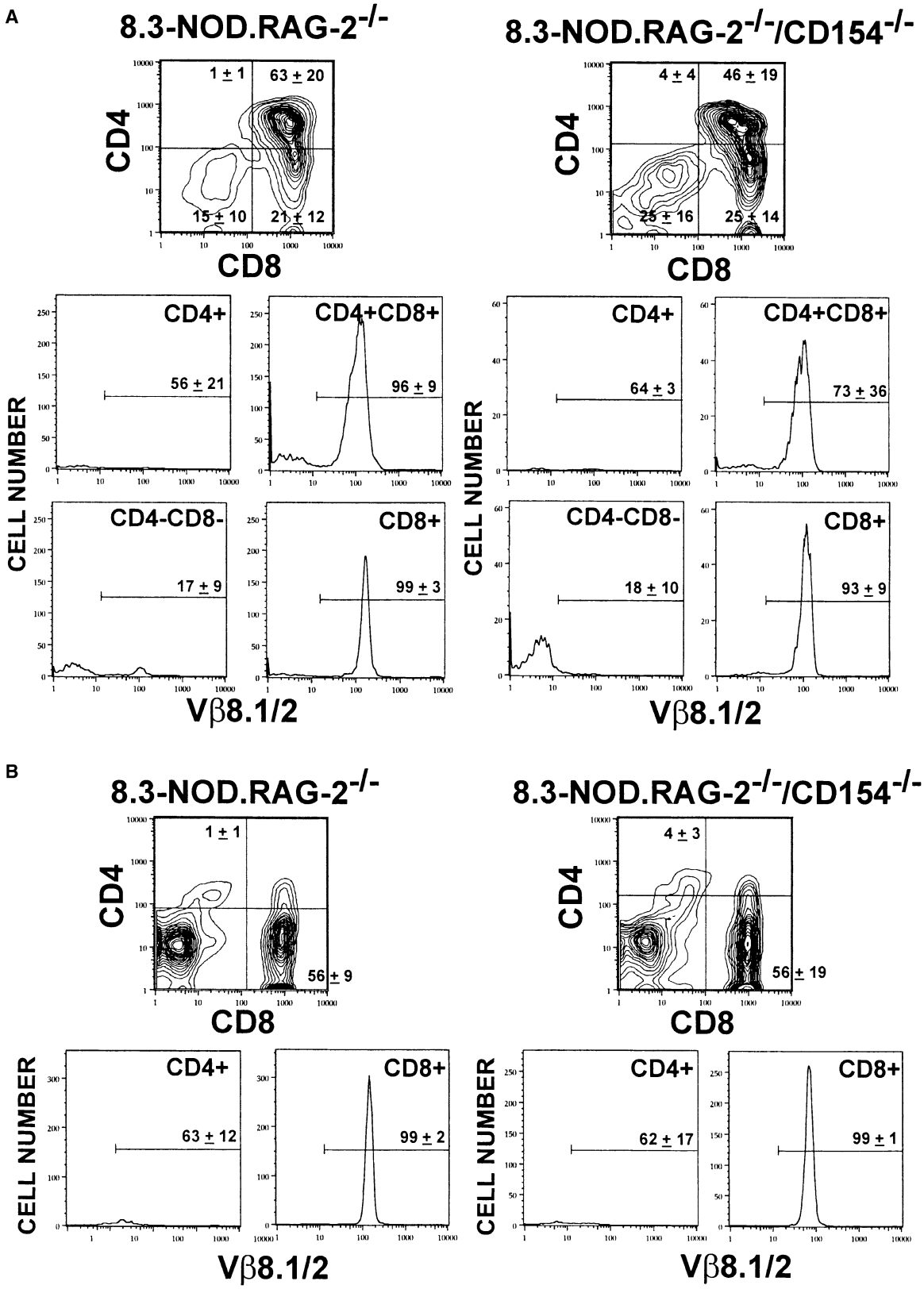


Figure 4. Development of *CD154*$^{+}$ and *CD154*$^{-/-}$ 8.3-CD8^{+} T Cells in Monoclonal T Cell NOD Mice

CD4, CD8, and Vβ8.1/8.2 profiles of thymocytes (A) and splenocytes (B) from 8.3-NOD.*RAG-2*$^{-/-}$ (n = 7) and 8.3-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ mice (n = 13). Numerical values correspond to percentage ± SD of cells within each gate.

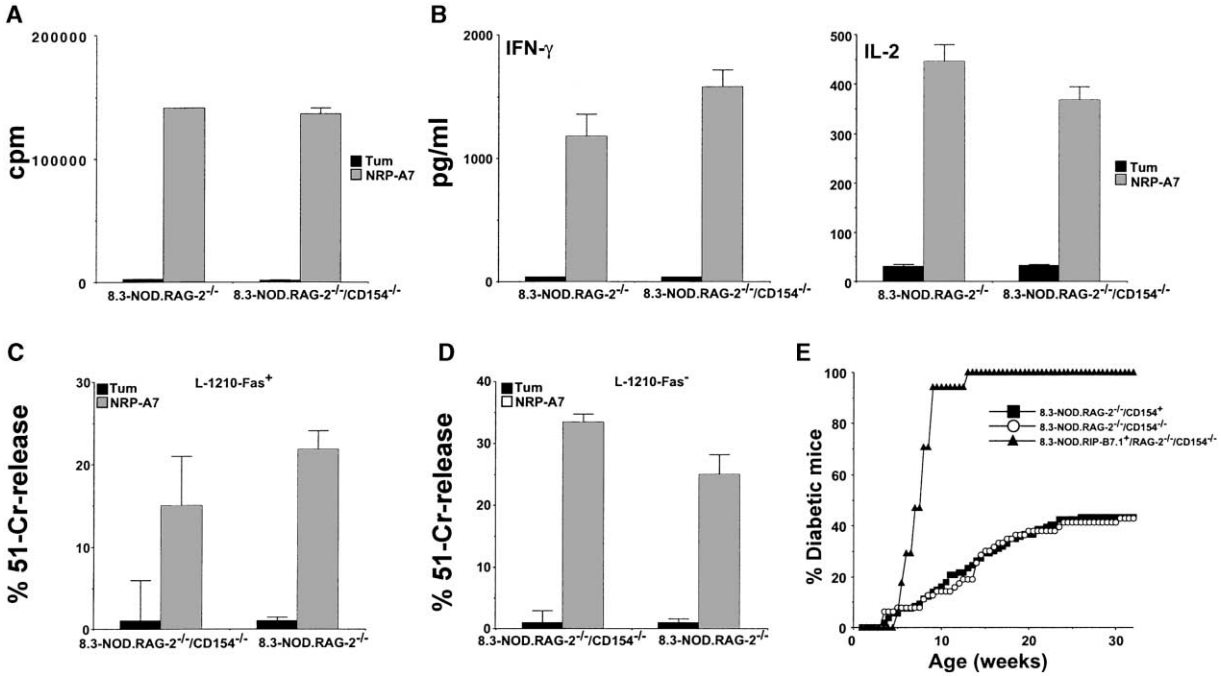


Figure 5. Function and Diabetogenicity of *CD154*$^{-/-}$ 8.3-CD8^{+} T Cells from Monoclonal T Cell NOD Mice

(A) Proliferation of splenic CD8^{+} T cells in response to NOD bone marrow-derived DCs pulsed with NRP-A7 or TUM (1 μM). CD8^{+} T cells (2×10^4) were incubated with 5×10^3 DCs for 3 days, pulsed with [^{3}H]-thymidine, harvested, and counted.

(B) Cytokine secretion by 8.3-CD8^{+} T cells in response to peptide-pulsed DCs.

(C and D) Cytotoxicity of NRP-A7-differentiated 8.3-CD8^{+} T cells against NRP-A7- or TUM-pulsed targets (L1210-Fas^{+} or L1210-Fas^{-}). Splenic 8.3-CD8^{+} T cells were challenged with NRP-A7-pulsed DCs as in (A) and expanded in the presence of rIL-2 for 4 additional days. The cells were used as effectors in ^{51}Cr-release assays at a 1:10 target:effector ratio. Bars show the standard error of the means.

(E) Cumulative incidence curves of diabetes in female 8.3-NOD.*RAG-2*$^{-/-}$/*CD154*$^{+}$ (n = 106), 8.3-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ mice (n = 63), and 8.3-NOD.*RIP-B7.1*$^{+}$/*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ mice (n = 18).

(Figure 5D). Unexpectedly, 8.3-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ mice developed diabetes with the same incidence and kinetics as 8.3-*NOD.RAG-2*$^{-/-}$/*CD154*$^{+}$ mice (Figure 5E), indicating that, unlike 4.1-CD4^{+} T cells, 8.3-CD8^{+} T cells do not need to express CD154 to differentiate into diabetogenic effectors in vivo.

CD154-Deficient 4.1-CD4^{+} T Cells Cannot Recognize Antigen in the Pancreatic Lymph Nodes of NOD Mice

Recruitment of diabetogenic T cells into islets is preceded by recognition of islet antigen on professional APCs in the pancreatic lymph nodes (Hoglund et al., 1999). To determine whether *CD154*-deficient 4.1-CD4^{+} T cells could undergo antigen-induced activation in vivo, we compared the ability of *CD154*$^{+}$ and *CD154*$^{-/-}$, CFSE-labeled 4.1-CD4^{+} T cells and 8.3-CD8^{+} T cells to proliferate in the peripheral lymphoid organs of nondiabetic NOD hosts. *CD154*$^{+}$ 4.1-CD4^{+} T cells, *CD154*$^{+}$ 8.3-CD8^{+} T cells, and *CD154*$^{-/-}$ 8.3-CD8^{+} T cells underwent several rounds of cell division exclusively in the pancreatic lymph nodes (Figure 6A). In contrast, *CD154*$^{-/-}$ 4.1-CD4^{+} T cells did not proliferate at all at this site (Figure 6A). Furthermore, whereas *CD154*$^{+}$ 4.1-CD4^{+} T cells upregulated both CD69 and CD44 in the pancreatic lymph nodes of the hosts within 6 days of transfer, *CD154*$^{-/-}$ 4.1-CD4^{+} T cells did not (Figures 6B and 6C). Thus, unlike 8.3-CD8^{+} T cells, 4.1-CD4^{+} T cells need to express CD154 to undergo antigen-driven activation and proliferation in the pancreatic lymph nodes.

Constitutive Expression of Transgenic B7.1 Molecules in β Cells Enhances the Diabetogenic Activity of *CD154*-Deficient 8.3-CD8^{+} but Not 4.1-CD4^{+} T Cells

To ascertain whether *CD154*-deficient 4.1-CD4^{+} T cells did not trigger diabetes because they were unable to elicit costimulatory activity in APCs, we studied the history of diabetes in 4.1-*NOD.RAG-2*$^{-/-}$/*CD154*$^{-/-}$ and 8.3-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ mice expressing a rat-insulin promoter (RIP) B7.1 transgene in β cells (Guerder et al., 1994). B7.1 molecules have costimulatory activity on CD8^{+} and CD4^{+} T cells both when provided in *cis* (i.e., when expressed on the antigen-loaded APC itself) and in *trans* (i.e., when expressed on bystander, nonantigen-bearing APCs) (Ding and Shevach, 1994; Mandelbrot et al., 2001). Whereas non-TCR-transgenic RIP-B7.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ mice did not develop diabetes (n = 0/27), expression of RIP-B7.1 molecules on β cells of 8.3-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ mice dramatically increased the incidence of diabetes and accelerated its onset (Figure 5E). Since most of the 8.3-NOD.*RAG-2*$^{-/-}$ mice that do not develop diabetes are also insulitis free, the above results indicated that presence of B7.1 molecules on β cells was somehow able to promote the accumulation of 8.3-CD8^{+} T cells in islets. Expression

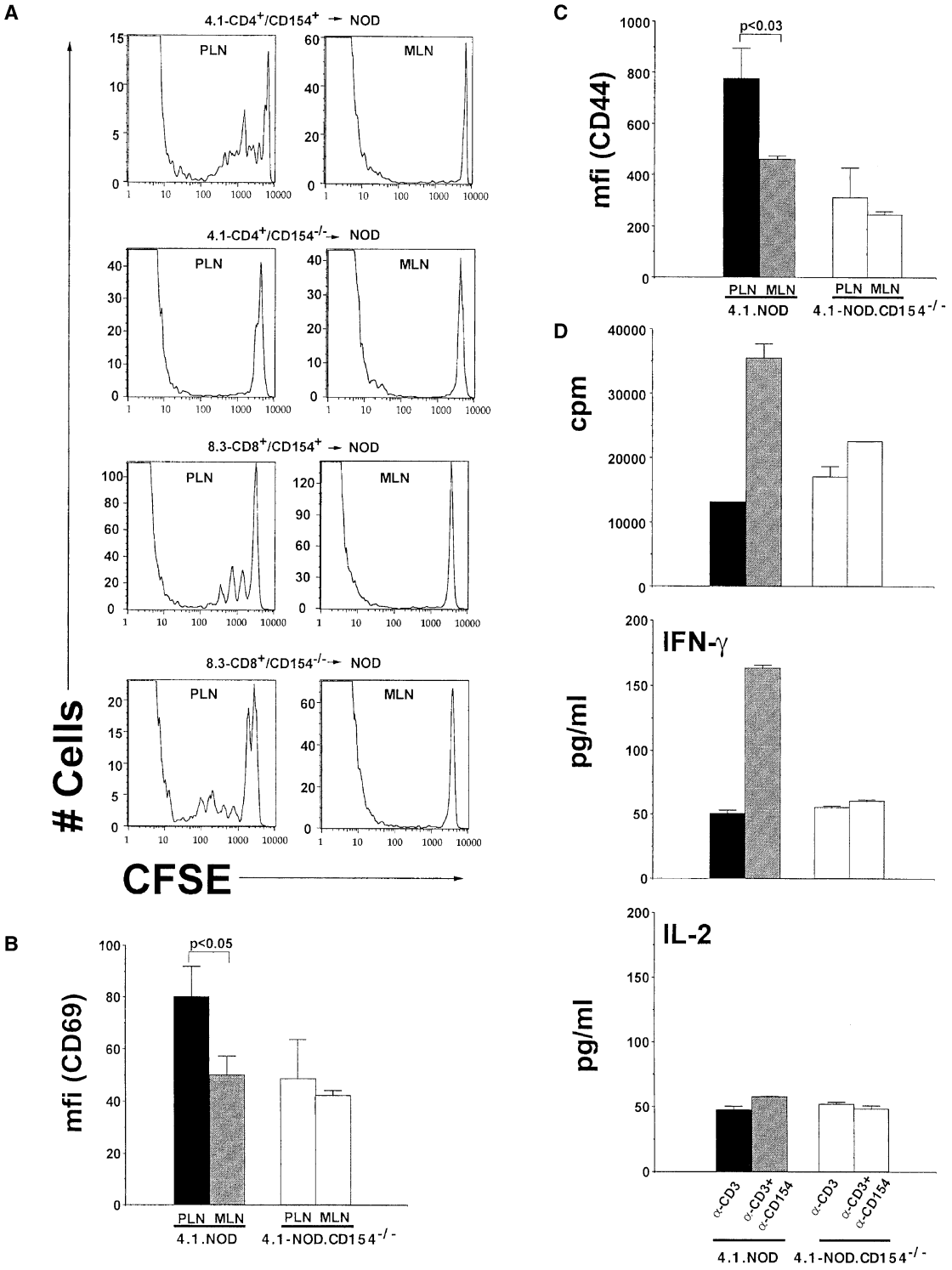


Figure 6. CD154-Dependent Activation of 4.1-CD4+ T Cells in the Pancreatic Lymph Nodes of NOD Mice and Costimulation of In Vitro 4.1-CD4+ T Cell Responses by CD154

(A) Naive splenic T cells from the corresponding transgenic mice were labeled with CFSE and injected i.v. into NOD hosts. The hosts were sacrificed 6 days later, and their pancreatic lymph nodes were examined for the presence of proliferating CFSE+ T cells by flow cytometry.

(B and C) Fluorescence intensity for CD69 and CD44 on *CD154*+ 4.1-CD4+ versus *CD154*−/− 4.1-CD4+ CFSE-labeled cells in the pancreatic (PLN) and mesenteric lymph nodes (MLN) 6 days after transfer into NOD hosts (n = 3–9 mice/group).

(D) Proliferation of and cytokine secretion by splenic CD4+ T cells (2×10^5) from 4.1-NOD and 4.1-NOD.*CD154*−/− mice in response to Dynabeads coated with anti-CD154 (56.7 ng/6×10^5 beads) and/or anti-CD3ε mAbs (0.28 ng/6×10^5 beads) mAbs. The cultures were pulsed with [^{3}H]-thymidine at 48 hr and harvested 18 hr later. The cytokine contents in the supernatants were evaluated at 48 hr.

Table 1. Adoptive T Cell Transfer and mAb/CpG Treatment Experiments in 4.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ Versus 8.3-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ Mice

Host (n)	Group	CD4+ T Cells, DCs, CpG DNA, and/or FGK45 mAb[a]	Diabetes Incidence (%)[b]	P Values[c]	Insulitis Score (n)	P Values[d]
8.3-NOD.*RAG-2*$^{-/-}$/*CD154*$^{+}$ (73)	1	–	27/73 (42.8%)	–	0.7 ± 0.8 (6)	–
8.3-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ (106)	2	–	46/106 (43.4%)	versus 1, NS	0.9 ± 0.9 (4)	–
8.3-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ (23)	3	CD4^{+} T cells	19/23 (82.6%)	versus 1 or 2, $p < 0.001$	ND	–
8.3-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ (19)	4	CpG DNA	17/19 (89.5%)	versus 1 or 2, $p < 0.001$	ND	–
8.3-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ (5)	5	Agonistic anti-CD40 mAb (FGK45)	5/5 (100%)	versus 1 or 2, $p < 0.015$	ND	–
8.3-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ (3)	6	Control mAb (anti-B220)	1/3 (33.3%)	versus 5, $P < 0.05$	ND	–
NOD.*RAG-2*$^{-/-}$/*CD154*$^{+}$ (7)	7	CD4^{+} T cells	0/7	versus 3, $p < 0.0001$	0 (3)	–
NOD.*RAG-2*$^{+}$/*CD154*$^{-/-}$ (6)	8	Agonistic Anti-CD40 mAb (FGK45)	0/6	versus 3, $p < 0.0001$	ND	–
4.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{+}$ (123)	9	–	84/123 (68%)	–	3.34 ± 0.1 (2)	–
4.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{+}$ (5)	10	DCs + CpG DNA	5/5 (100%)	–	ND	–
4.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ (47)	11	–	0/47 (0%)	versus 9 < 0.0001	0.13 ± 0.2 (3)	–
4.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ (15)	12	CD4^{+} T cells	1/15 (6.7%)	versus 9 < 0.0001	0.92 ± 0.3 (6)	versus 9, $p < 0.05$
4.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ (6)	13	CD4^{+} T cells + CpG DNA	0/6 (0%)	versus 9 < 0.0001	1.63 ± 0.972 (4)	–
4.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ (5)	14	CpG DNA	0/5 (0%)	versus 9 < 0.002	0 (2)	–
4.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ (6)	15	Agonistic anti-CD40 mAb (FGK45)	1/6 (17%)	versus 9 < 0.01	2.3 ± 0.1 (2)	versus 9, $p < 0.05$
4.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ (6)	16	Control mAb (Anti-B220)	0/6 (0%)	versus 9 < 0.0006	ND	–
4.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ (6)	17	DCs + CpG DNA[e]	0/6 (0%)	versus 9 < 0.0006	ND	–

[a] 1.5×10^7 purified splenic CD4^{+} or 8×10^6 CD8^{+} T cell-depleted splenocytes (no differences) from prediabetic NOD mice into 8- to 12-week-old hosts. Some mice were treated with one i.v. injection of CpG DNA (10 nmoles) or three consecutive i.v. or i.p. injections of mAb (100 μg/injection) 3–4 days apart. Mice from group 17 were treated with two i.v. injections of 1–2 $\times 10^6$ bone marrow-derived dendritic cells (DCs) (activated in vitro by overnight culture in 1 μg/ml LPS) and CpG DNA (10 nmoles i.p.) 7 days apart into 4- to 8-week-old hosts. Group 10 hosts were 3-week-old, and all developed diabetes within 24 hr ($n = 2$) or 48 hr ($n = 3$) after the first injection of activated DCs and CpG.

[b] Hosts were followed for 9 (group 17) or >10 weeks after treatment (all other groups).

[c] Chi-square.

[d] Mann-Whitney U test.

[e] These mice (group 17) were treated with two doses of FGK45 (100 μg/injection, 3 days apart, i.p.) 6 weeks after receiving DC + CpG and then followed for additional 3 weeks. None developed diabetes.

of the RIP B7.1 transgene in 4.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{+}$ mice also increased the incidence of 4.1-CD4^{+} T cell-induced diabetes (from 68% to 100%; data not shown). In contrast, none of the 34 4.1/RIP-B7.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ mice that were followed developed diabetes (Figure 3A). Adoptive transfer experiments of in vitro-activated, B7-expressing DCs into mice followed by agonistic anti-CD40 mAb and/or CpG DNA treatment confirmed that this was not the result of inadequate exposure of 4.1-CD4^{+} T cells to B7 molecules on islet cells (see below). Therefore, expression of costimulatory B7.1 molecules on β cells can promote the accumulation of *CD154*-deficient 8.3-CD8^{+} T cells and *CD154*-competent 4.1-CD4^{+} T cells in islets but cannot drive the recruitment of *CD154*-deficient 4.1-CD4^{+} T cells to the site.

CD154-Deficient 4.1-CD4^{+} T Cells Cannot Respond to CD4^{+} T Cell-help

Splenic CD4^{+} T cells from prediabetic NOD mice significantly enhance the diabetogenic activity of naive 8.3-CD8^{+} T cells upon adoptive transfer into 8.3.NOD.*RAG-2*$^{-/-}$ hosts (Verdaguer et al., 1997). To determine whether *CD154*$^{-/-}$ 8.3-CD8^{+} and *CD154*$^{-/-}$ 4.1-CD4^{+} T cells can respond to exogenous T cell help, we transfused CD4^{+} splenocytes from wild-type NOD mice into NOD.*RAG-2*$^{-/-}$/*CD154*$^{+}$, 8.3-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$, and 4.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ hosts. NOD CD4^{+} splenocytes did not even induce insulitis in lymphocyte-deficient NOD.*RAG-2*$^{-/-}$/*CD154*$^{+}$ females (Group 7 in Table 1) but significantly increased the incidence of diabetes in 8.3-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ mice (Groups 2 and 3 in Table 1). This T helper effect was CD154 dependent, since it did not occur in 8.3-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ hosts that were transfused with splenic CD4^{+} T cells from NOD.*CD154*$^{-/-}$ donors (data not shown). In contrast, while adoptively transferred 4.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ mice developed mild peri-insulitis, they remained diabetes free for at least 10 weeks after transfer (Group 12 in Table 1). These results thus indicated that, unlike *CD154*$^{-/-}$ 8.3-CD8^{+} T cells, *CD154*$^{-/-}$ 4.1-CD4^{+} T cells cannot respond to autoantigen-loaded APCs activated by *trans* CD154 signals.

CD40 Crosslinking In Vivo Triggers Diabetes in 8.3-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ Mice but Not in 4.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ Mice

CTL responses against viruses are impaired in *CD154*$^{-/-}$ mice but develop normally upon treatment of the mice with agonistic anti-CD40 mAbs, indicating that CD40 crosslinking can overcome the need for CD154 in the generation of CTL responses in vivo (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998). To determine whether ligation of CD40 on APCs could enhance the diabetogenic activity of *CD154*$^{-/-}$ 8.3-CD8^{+} T cells and/or restore the diabetogenic potential of *CD154*$^{-/-}$ 4.1-CD4^{+} T cells, we treated 8.3-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ and 4.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ mice with the agonistic anti-CD40 mAb FGK45. FGK45 mAb treatment dramatically increased the incidence of diabetes in 8.3-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ mice when compared to untreated or anti-B220 mAb-treated controls (Table 1, Groups 5, 2, and 6, respectively). In contrast, although FGK45 treatment increased the severity of insulitis in 4.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ mice (Group 15 in Table 1), it did not promote diabetogenesis when compared to untreated and anti-B220 mAb-treated controls (Table 1, Groups 11 and 16, respectively).

Systemic Activation of APCs with Bacterial CpG DNA Accelerates Diabetogenesis in 8.3-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ but Not 4.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ Mice

CpG oligodeoxynucleotides bind to the Toll-like Receptor 9 on DCs, B cells, and macrophages, inducing their activation (Hemmi et al., 2000). To determine whether systemic activation of APCs with CpG DNA could uncouple 4.1-CD4^{+} T cell activation from CD154, we compared the effects of CpG DNA on the natural history of diabetes in 8.3-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ and 4.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ mice. CpG DNA clearly enhanced the diabetogenic potential of *CD154*$^{-/-}$ 8.3-CD8^{+} T cells (Table 1, Group 4) but was unable to restore the diabetogenic potential of *CD154*$^{-/-}$ 4.1-CD4^{+} T cells, even when given in combination with NOD splenic CD4^{+} T cells (Table 1, Groups 14 and 13, respectively). This inability of CpG and FGK45 to elicit the activation of *CD154*$^{-/-}$ CD4^{+} T cells in vivo was confirmed by treatment of the mice with both activated DCs plus FGK45 and/or CpG DNA. Whereas all the 4.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{+}$ mice that received activated DCs and CpG developed diabetes within 2 days after the first injection, diabetes did not develop in any of 6 4.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ mice that received two doses of the same treatment (Group 10 versus 17 in Table 1). Furthermore, these mice remained diabetes free for at least 3 weeks following two consecutive i.p. injections of FGK45 mAb given 6 weeks after the first DC+CpG injection.

Costimulation of In Vitro 4.1-CD4^{+} T Cell Responses by CD154

It has been shown that CD154 has the potential to costimulate the proliferative and cytokine secretion activity of human CD4^{+} T cells in vitro (Blair et al., 2000). In addition, ligation of CD154 on human Jurkat T cells and mouse splenic T cells induces the activation of both JNK1 and p38 MAPK (Brenner et al., 1997), which play critical roles in T cell proliferation and cytokine production (Kane et al., 2000). To determine whether CD154 signaling costimulates 4.1-CD4^{+} T cell responses, we tested the ability of CD154 crosslinking to potentiate the agonistic activity of suboptimal concentrations of anti-CD3ε mAb coated on beads. Anti-CD154 enhanced the ability of anti-CD3 mAb to trigger the proliferation and the secretion of IFN-γ by 4.1-CD4^{+} T cells (Figure 6D). No significant differences in IL-2 secretion were noted. These effects were CD154-dependent as they were not seen with *CD154*$^{-/-}$ 4.1-CD4^{+} T cells (Figure 6D). Thus, as previously proposed for human T cells, CD154 can costimulate murine 4.1-CD4^{+} T cell responses in vitro.

Discussion

Diabetogenesis results from well-orchestrated, but ill-defined, collaborations between autoreactive CD4^{+} and CD8^{+} T cells and professional APCs (Delovitch and Singh, 1997). Previous studies have shown that *CD154*-

deficient NOD mice are completely resistant to both diabetes and insulitis, suggesting a role for CD154/CD40 interactions in the initiation of the disease process (Green et al., 2000). Here we have investigated the role of CD154/CD40 interactions and APC activation in the recruitment of diabetogenic $CD4^+$ and $CD8^+$ T cells in spontaneous autoimmune diabetes, using disease-relevant, informative reductionist systems. Our results demonstrate that, despite the existence of CD154-independent pathways of APC activation and T-T collaboration in vivo (Bachmann et al., 1999; Green and Flavell, 1999; Ruedl et al., 1999; Lu et al., 2000), CD154 triggering is a key event in the activation of diabetogenic $CD4^+$ (but surprisingly not $CD8^+$) T cells. Importantly, the data show that the inability of *CD154*-deficient 4.1-$CD4^+$ T cells to trigger diabetes cannot be overcome by strategies that bypass the need for CD40 ligation and thus demonstrate the existence of a novel, CD154-dependent pathway of $CD4^+$ T cell activation that is independent of CD40-mediated activation of APCs.

Abrogation of CD154 expression rendered 4.1-$CD4^+$ T cells unable to proliferate against islet antigen in the pancreatic lymph nodes and hence to accumulate in islets, without impairing their positive selection in the thymus or their ability to proliferate in response to islet antigen ex vivo. This inability of *CD154*$^{-/-}$ 4.1-$CD4^+$ T cells to trigger insulitis is reminiscent of the inability of myelin basic protein (MBP)-specific TCR-transgenic *CD154*$^{-/-}$ $CD4^+$ T cells to undergo priming in antigen-challenged mice despite being able to proliferate in response to MBP in vitro (Grewal et al., 1996). However, the underlying mechanisms appear to be different. Whereas *CD154*$^{-/-}$ MBP-reactive $CD4^+$ T cells primed efficiently in the presence of $B7.1^+$ APCs (Grewal et al., 1996), *CD154*$^{-/-}$ 4.1-$CD4^+$ T cells remained quiescent in mice transfused with TNFα/LPS-activated DCs, in mice expressing B7.1 molecules on β cells, or in mice treated with potent DC activators. CpG DNA and agonistic anti-CD40 mAbs induce costimulatory activity on DCs and/or uncouple $CD8^+$ T cell activation and CTL differentiation from the need of $CD4^+$ T helper cells in different models (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998; Schuurhuis et al., 2000). Furthermore, they promote the migration of antigen-loaded DCs into draining lymph nodes (Moodycliffe et al., 2000) and elicit the production of high levels of IL-12 by these cells (Cella et al., 1996; Koch et al., 1996; Hemmi et al., 2000). Both CpG DNA and agonistic anti-CD40 mAb dramatically enhanced the pathogenicity of *CD154*$^{-/-}$ 8.3-$CD8^+$ T cells but were surprisingly ineffective at modifying the pathogenicity of *CD154*$^{-/-}$ $CD4^+$ cells, even in the presence of *CD154*-competent $CD4^+$ T helper cells. Splenic $CD4^+$ T cells from prediabetic NOD mice (containing autoreactive T helper cells) potentiate the insulitogenic activity of *CD154*$^{-/-}$ 8.3-$CD8^+$ T cells in a CD154-dependent manner, possibly by inducing the activation and maturation of DCs, as in other models (Caux et al., 1994; Cella et al., 1996; Bennett et al., 1998; Borrow et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998; Buhlmann et al., 1999). The inability of $CD4^+$ T helper cells, CpG DNA, and agonistic anti-CD40 mAb to activate *CD154*$^{-/-}$ $CD4^+$ T cells is consistent with the idea that neither ligation of CD40 nor other APC activatory receptors, such as TRANCE-R, 4-1BB-ligand, or OX-40-ligand by $CD4^+$ T helper cells (den Haan and Bevan, 2000) can bypass the requirement for CD154 engagement. Thus, when taken together, these data show that CD154 triggering is indispensable for the activation of 4.1-$CD4^+$ T cells.

Crosslinking of CD154 potentiated in vitro 4.1-$CD4^+$ T cell responses induced by suboptimal concentrations of anti-CD3 mAb in a CD154-dependent manner, indicating that CD154 can indeed deliver intracellular signals in 4.1-$CD4^+$ T cells. This is in agreement with previous in vitro studies that supported a costimulatory role for CD154 signaling in both human and murine T cells. Anti-CD154 mAbs, for example, potentiate anti-CD3-induced proliferation of human $CD4^+$ T cells in vitro (Blair et al., 2000; Blotta et al., 1996), induce expression of CD54 and CD44H (Shinde et al., 1996; Gurunathan et al., 1998), and elicit production of IFN-γ, TNFα, and IL-10 by these T cells (Blair et al., 2000). Furthermore, ligation of CD154 on both human and mouse T cells induces the activation of both JNK1 and p38 MAPK (Brenner et al., 1997), which are involved in signal integration and cytokine production during T cell costimulation (Kane et al., 2000). The fact that 4.1-$CD4^+$ T cells can neither proliferate nor upregulate activation markers in vivo in the absence of CD154 is consistent with all of these in vitro observations and provide a mechanistic explanation for the complete resistance of 4.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ mice to diabetes development. Furthermore, they account for the inability of transgenic RIP-B7.1 molecules, in vitro-activated DCs, agonistic anti-CD40 mAb, and CpG DNA to induce the activation of *CD154*$^{-/-}$ 4.1-$CD4^+$ T cells in our system. Whether ligation of CD154 contributes to the recruitment of effector 4.1-$CD4^+$ T cells by inducing some of the responses described above remains to be determined but, with the exception of IL-10 (Moritani et al., 1996), they are all good candidates. Although anti-CD3/CD154 crosslinking in human T cells in vitro ultimately results in apoptosis (Blair et al., 2000), ligation of CD154 on 4.1-$CD4^+$ T cells in vivo does not occur in isolation and thus its proapoptotic effect in human T cells in vitro is likely to be overridden by other costimulatory signals.

8.3-$CD8^+$ T cells do not efficiently accumulate into pancreatic islets in the absence of $CD4^+$ T cells (Verdaguer et al., 1997). However, 8.3-$CD8^+$ T cells trigger a delayed, T helper-independent form of diabetes in 8.3-NOD.*RAG-2*$^{-/-}$ mice and thus their reliance on $CD4^+$ T helper cells is not absolute (Verdaguer et al., 1997). Data shown here have indicated that although CD154/CD40 interactions are indispensable for the activation of 4.1-$CD4^+$ T cells and for the T helper-dependent activation of 8.3-$CD8^+$ T cells, they are totally dispensable for the $CD4^+$ T cell-independent diabetogenic activity of 8.3-$CD8^+$ T cells. Although surprising, this was not a totally unexpected observation, as $CD8^+$ T cells are less dependent on costimulation than $CD4^+$ T cells, particularly in alloreactive and antiviral immune responses (Bachmann et al., 1999; Newell et al., 1999; Ruedl et al., 1999; Trambley et al., 1999; Kishimoto et al., 2000; Whitmire and Ahmed, 2000). On the other hand, activation of certain $CD8^+$ T cell specificities requires the presence of CD154 on the $CD8^+$ T cells themselves (Lefrancois et al., 1999). Since 8.3-$CD8^+$ T cells can recognize islet antigen in the pancreatic lymph nodes in the absence

of CD4+ T cells, our data indicate that there must be some constitutive processing of β cell autoantigens and subsequent migration of antigen-loaded DCs to the pancreatic lymph nodes in the absence of a T cell-mediated insult. In turn, this implies that in 8.3-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ mice, 8.3-CD8+ T cells must somehow be able to induce costimulatory activity on DCs via molecules other than CD154, such as TRANCE or TNF-α (Bachmann et al., 1999; Green and Flavell, 1999; Green et al., 2000). It should be noted that 8.3-CD8+ T cells secrete abundant levels of TNFα upon antigen recognition (Verdaguer et al., 1997) and that TNFα promotes CD154-independent presentation of antigen to CD8+ T cells (Green et al., 2000). Thus, 8.3-CD8+ T cell-derived TNFα is a likely candidate as a costimulator-inducing signal.

The complete absence of insulitis in *CD154*$^{-/-}$ NOD and anti-CD154 mAb-treated NOD mice (Balasa et al., 1997; Green et al., 2000) has conclusively shown that CD154/CD40 interactions play a critical role in the initiation of autoimmune diabetes. However, because of the cellular and molecular complexity of the diabetogenic autoimmune response in wild-type NOD mice, it has not been possible to distinguish between a role for CD40-CD154 interactions in the activation of CD4+ T cells, CD8+ T cells, and/or APCs. Our observation that CD154 triggering is essential for the recruitment of 4.1-CD4+ T cells, coupled with the exquisite diabetogenic activity of 4.1-CD4+ T cells, and the CD154-dependent, T helper-assisted recruitment of CD8+ T cells suggests that diabetogenesis in wild-type NOD mice requires signaling through CD154 on 4.1-like CD4+ T cells. Although it remains to be determined whether CD154 triggering is involved in the activation of other CD4+ T cell responses, our observations support a model for the initiation of diabetogenesis in which activation of insulitogenic CD4+ T cells requires CD154 triggering, independently of CD40 ligation or APC activation. This initial step would be followed by insulitis, recruitment of diabetogenic CD8+ T cells, avidity maturation (assisted by CD4+ T cells), and β cell destruction.

Experimental Procedures

Mice

8.3-NOD.*RAG-2*$^{-/-}$ and 4.1-NOD.*RAG-2*$^{-/-}$ mice have been described (Verdaguer et al., 1997). RIP-B7.1-NOD.*scid* mice (Guerder et al., 1994) were obtained from D. Serreze (The Jackson Lab, Bar Harbor, ME). NOD.*CD154*$^{-/-}$ mice were produced by backcrossing the CD154 mutation of C57BL/6.*CD154*$^{-/-}$ mice (The Jackson Lab) (Renshaw et al., 1994) onto NOD/Lt mice for nine generations. Mice were PCR typed for microsatellite polymorphisms linked to *Idd2*, *Idd3*, *Idd4*, *Idd5*, *Idd7*, *Idd8/12*, *Idd9*, *Idd14*, and *Idd15* to confirm the absence of C57BL/6-derived, diabetes-protective alleles at known *Idd* loci. NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$, RIP-B7.1-NOD.*CD154*$^{-/-}$, 8.3- or 4.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$, 8.3- or 4.1-NOD.*RAG-2*$^{+}$/*CD154*$^{-/-}$, 8.3/RIP-B7.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$, and 4.1/RIP-B7.1-NOD.*RAG-2*$^{-/-}$/*CD154*$^{-/-}$ mice were produced by intercrossing heterozygous and homozygous mutant/transgenic mice and by screening the offspring for inheritance of the transgenes and mutations by PCR of tail DNA. All mice were housed under specific pathogen-free conditions.

Diabetes

Diabetes was monitored by measuring urine glucose levels with Diastix (Miles, Ontario, Canada) twice weekly. Animals were considered diabetic after two consecutive readings ≥3+.

Cell Lines, Antibodies, and Flow Cytometry

L2120-Fas+ and L2120-Fas− cells were from P. Goldstein (CNRS, Marseille, France). H-2K^{d}-transfected RMA-S cells (RMA-SK^{d}) were from M. Bevan (University of Washington, Seattle, WA). The FGK45 (anti-CD40) hybridoma was from A. Rolink (Basel Institute for Immunology, Basel, Switzerland). Anti-CD8α-PE (53-6.7), anti-CD4-FITC/biotin (IM7), anti-CD69-PE (H1.2F3), anti-CD44-biotin (IM7), anti-CD154 (MR1), anti-Vβ11-FITC (RR3-15), anti-Vβ8.1/8.2-FITC (MR5-2), anti-I-A$\beta^{k/g7}$-biotin (10-3.6), anti-CD45R/B220-biotin or -PerCP (RA36B2), anti-CD11c-biotin (HL3), anti-CD11b-biotin (M1/70), anti-B7.1-biotin (16-10A1), anti-B7.2-biotin (GL1), and anti-H-2K^{d}-PE (SF1-1.1) were from PharMingen (San Diego, CA). Mouse IgG-absorbed goat anti-rat IgG-FITC/biotin, and goat anti-mouse IgG-FITC and Streptavidin-PerCP were from Caltag (San Francisco, CA) and Becton-Dickinson (San Jose, CA), respectively. Carboxyfluorescein diacetate succinimidyl ester (CFSE) was from Molecular Probes (Eugene, OR).

Peptides

The peptides NRP-A7 and TUM were prepared using FMOC chemistry, purified by rpHPLC to >90% purity, and sequenced by mass spectrometry (Chiron Technologies, San Diego, CA).

Bone Marrow-Derived DCs and Islet Cells

Bone marrow-derived DCs were prepared by culturing bone marrow cells in the presence of rmGM-CSF and rmIL4 (5 ng/ml) (Lutz et al., 1999). Resulting cells were >70% CD11c+, CD11b−, I-A^{g7+}, K^{d+}, CD40+, B7.1+, and B7.2+. Activation of DCs for in vivo experimentation was induced by overnight culture in the presence of LPS (1 μg/ml). Mice were treated with two injections of 1–2 × 10^{6} DCs (i.v.) and CpG DNA (10 nmoles; i.p.) 7 days apart. To prepare islet cells, pancreatic islets were isolated by collagenase digestion of the pancreas, purified on Ficoll gradients, handpicked, and disrupted into single-cell suspensions using Cell Dissociation Buffer (Gibco-BRL, Gaithesburg, MD).

Generation of 8.3-CD8+ CTL

Spleen cells were adjusted to 2 × 10^{4} CD8+ T cells/100 μl of RPMI 1640 containing 10% heat-inactivated fetal bovine serum, stimulated with NRP-A7 (0.0001 to 1 μM) or TUM-pulsed (1 μM) bone marrow-derived DCs (5 × 10^{3} cells/well) for 3–4 days, and expanded in the presence of 0.5 U/ml of rIL-2 (Takeda, Osaka, Japan) for 7–10 days. The growing cells (>95% CD8+ pure) were used as effectors in cytotoxicity assays.

^{51}Cr-Release Assays

L1210-Fas+ and L1210-Fas− cells were labeled with [^{51}Cr]-sodium chromate for 2 hr at 37°C, washed, seeded at 10^{4} cells per 100 μl/well, pulsed with NRP-A7 or TUM (1 μM) for 1 hr at 37°C, and used as target cells in ^{51}Cr-release assays. Effector cells were added to each well in duplicate at a 1:10 target:effector ratio. Plain medium or 1% Triton X-100 was added to sets of target cells for examination of spontaneous and total cell lysis, respectively. The plates were incubated at 37°C for 8 hr, and the supernatants were collected for determination of specific ^{51}Cr-release.

Proliferation and Cytokine Secretion Assays

Splenic 8.3-CD8+ T cells (2 × 10^{4}/well) were incubated, in duplicate, with NRP-A7 (0.0001 to 1 μM) or TUM-pulsed (1 μM) bone marrow-derived DCs (2 × 10^{4}/well) for 3 days at 37°C in 5% CO_2. The proliferative activity of *CD154*$^{-/-}$ 4.1-CD4+ T cells was assessed by culturing splenic 4.1-CD4+ T cells (2 × 10^{4}/well) with irradiated islet cells from NOD or NOD.*CD154*$^{-/-}$ mice (1 × 10^{5}/well) as a source of antigen in the absence of exogenous APCs. Alternatively, CD4+ T cells (2 × 10^{5}/well) were cultured for 3 days with 0.5 μM polyurethane-coated, tosyl-activated beads coated with anti-CD154 and/or an anti-CD3ε mAbs as per the manufacturer's instructions (Dynal; 6 × 10^{5} beads/well). In brief, 3.4 × 10^{7} beads were incubated with 10 μg anti-CD154 and/or 0.05 μg anti-CD3 mAbs in a total volume of 43 μl, washed, and used. The chosen concentration of anti-CD3 mAb corresponded to the lowest concentration that induced T cell responses above background. Cultures were pulsed with 1 μCi of [^{3}H]-thymidine during the last 18 hr of culture.

Cytokine Secretion

Naive splenic 8.3-CD8$^+$ T cells (2×10^4/well) were incubated with NRP-A7- or TUM-pulsed (1 μM) NOD DCs (5×10^3/well), or mAb-coated beads in 96-well plates for 48 hr at 37°C. The supernatants (100 μl/well) were assayed for IL-2, IL-4, and/or IFN-γ content by ELISA using commercially available kits (R&D Systems, Minneapolis, MN).

Cytokine Profile of Intra-Islet T Cells

Freshly isolated islets and islet-associated T cells (2×10^4) were activated with phorbol-myristate acetate (PMA,10 ng/mL) and ionomycin (250 ng/mL) for 12 hr at 37°C, 5% CO_2. The supernatants were assayed for IL-2, IL-4, and IFN-γ content by ELISA (R&D Systems, Minneapolis, MN).

Adoptive T Cell Transfer

To measure the proliferative activity of 8.3-CD8$^+$ and 4.1-CD4$^+$ T cells in vivo, splenic 8.3-CD8$^+$ or 4.1-CD4$^+$ T cells were purified with anti-CD8 or anti-CD4 mAb-coated microbeads (MiniMACS, Miltenyi Biotec), labeled with CFSE and injected into the tail veins of 8- to 10-week-old NOD hosts (10^7 cells). Hosts were sacrificed 6 days later and their pancreatic and mesenteric lymph nodes examined for presence of CSFE-positive cells and for the levels of CD69 and CD44 on the transfused T cells. To transfer CD4$^+$ T cells, splenocytes from NOD or NOD.*CD154*$^{-/-}$ mice were depleted of CD8$^+$ T cells and B cells or subjected to positive selection with anti-CD4 mAb-coated magnetic microbeads. The cells (1.5×10^7) were injected into the tail veins of recipient mice, and the mice were followed for diabetes development.

Agonistic Anti-CD40 mAb and CpG DNA Treatment

Anti-CD40 and anti-B220 mAbs were purified from MAP-tested hybridoma culture supernatant by affinity chromatography. Mice received three injections of 100 μg of mAb i.p. or i.v. 3 days apart. Phosphorothioate-stabilized CpG oligo (TCCATGACGTTCCT GATGCT) was from MWG Biotech (Charlotte, NC). Mice were treated with one i.p injection of DNA (10 nmoles) and followed for diabetes. Some mice were injected with CD4$^+$ T cells 24 hr after oligodeoxynucleotide treatment.

Histopathology

Pancreata were fixed in formalin, embedded in paraffin, sectioned at 4.5 μM, stained with hematoxylin and eosin, and examined for inflammation. The degree of insulitis was evaluated by scoring 15–30 islets/mouse using the following criteria: 0, normal islet; 1, peri-insulitis; 2, mononuclear cell infiltration in $<$25% of the islet; 3, mononuclear cell infiltration in 25%–50% of the islet; 4, $>$50% of the islet infiltrated.

Statistical Analyses

Data was compared by Mann-Whitney U test or χ^2.

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References

Amrani, A., Verdaguer, J., Serra, P., Tafuro, S., Tan, R., and Santamaria, P. (2000a). Progression of autoimmune diabetes driven by avidity maturation of a T-cell population. Nature *406*, 739–742.

Amrani, A., Verdaguer, J., Thiessen, S., Bou, S., and Santamaria, P. (2000b). IL-1α, IL-1β, and IFN-γ mark beta cells for Fas-dependent destruction by diabetogenic CD4+ T lynphocytes. J. Clin. Invest. *105*, 459–468.

Anderson, B., Park, B.J., Verdaguer, J., Amrani, A., and Santamaria, P. (1999). Prevalent CD8(+) T cell response against one peptide/MHC complex in autoimmune diabetes. Proc. Natl. Acad. Sci. USA *96*, 9311–9316.

Bachmann, M., Wong, B., Josien, R., Steinman, R., Oxenius, A., and Choi, Y. (1999). TRANCE, a tumor necrosis factor family member critical for CD40 ligand-independent T helper cell activation. J. Exp. Med. *189*, 1025–1031.

Balasa, B., Krahl, T., Pastone, G., Lee, J., Tisch, R., McDevitt, H., and Sarvetnick, N. (1997). CD40 ligand-CD40 interactions are necessary for the initiation of insulitis and diabetes in nonobese diabetic mice. J. Immunol. *159*, 4620–4627.

Bennett, S., Carbone, F., Karamalis, F., Flavell, R., Miller, J., and Heath, W. (1998). Help for cytotoxic T-cell responses is mediated by CD40 signalling. Nature *393*, 478–480.

Blair, P.J., Riley, J.L., Harlan, D.M., Abe, R., Tadaki, D.K., Hoffmann, S.C., White, L., Francomano, T., Perfetto, S.J., Kirk, A.D., and June, C.H. (2000). CD40 ligand triggers a short-term CD4(+) T cell activation response that results in secretion of immunoupmodulatory cytokines and apoptosis. J. Exp. Med. *191*, 651–660.

Blotta, M., Marshall, J., DeKruyff, R., and Umetsu, D. (1996). Cross-linking of the CD40-ligand on human CD4+ T lymphocytes generates a costimulatory signal that up-regulates IL-4 synthesis. J. Immunol. *156*, 3133–3140.

Borrow, P., Tough, D., Eto, D., Tishon, A., Grewal, I., Sprent, J., Flavell, R., and Oldstone, M. (1998). CD40 ligand-mediated interactions are involved in the generation of memory CD8+ CTL but are not required for the maintenance of CTL memory following virus infection. J. Virol. *72*, 7440–7449.

Brenner, B., Koppenhoefer, U., Grassme, H., Kun, J., Lang, F., and Gulbins, E. (1997). Evidence for a novel function of the CD40 ligand as a signaling molecule in T lymphocytes. FEBS Lett. *417*, 301–306.

Buhlmann, J., Gonzalez, M., Ginther, B., Paniskaltsis-Mortari, A., Blazar, B., Greiner, D., Rosini, A., Flavell, R., and Noelle, R. (1999). Sustained expansion of CD8+ T cells requires CD154 expression by Th cells in acute graft versus host disease. J. Immunol. *162*, 4373–4376.

Caux, C., Massacrier, C., Vanbervliet, B., Dubois, B., Kooten, C., Durrand, I., and Banchereau, J. (1994). Activation of human dendritic cells through CD40 crosslinking. J. Exp. Med. *180*, 1263–1272.

Cayabyab, M., Phillips, J., and Lanier, L. (1994). CD40 preferentially costimulates activation of CD4+ T lymphocytes. J. Immunol. *152*, 1523–1531.

Cella, M., Scheidegger, D., Palmer-Lehmann, K., Lane, P., Lanzavecchia, A., and Albert, G. (1996). Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T-cell stimulatory capacity: T-T help via APC activation. J. Exp. Med. *184*, 747–752.

Christianson, S., Shultz, L., and Leiter, E. (1993). Adoptive transfer of diabetes into immunodeficient NOD-scid/scid mice. Relative contributions of CD4+ and CD8+ T-cells from diabetic versus prediabetic NOD.NON-thy-1a donors. Diabetes *42*, 44–55.

Delovitch, T., and Singh, B. (1997). The nonobese diabetic mouse as a model of autoimmune diabetes: immune disregulation gets the NOD. Immunity *7*, 727–738.

den Haan, J.M., and Bevan, M.J. (2000). A novel helper role for CD4 T cells. Proc. Natl. Acad. Sci. USA *97*, 12950–12952.

DiLorenzo, T., Graser, R., Ono, T., Christianson, G., Chapman, H., Roopenian, D., Nathenson, S., and Serreze, D. (1998). MHC class I-restricted T cells are required for all but the end stages of diabetes development in nonobese diabetic mice and a use prevalent T cell receptor x chain gene rearrangement. Proc. Natl. Acad. Sci. USA *95*, 12538–12543.

Ding, L., and Shevach, E. (1994). Activation of CD4+ T-cells by delivery of the B7 costimulatory signal on bystander antigen-presenting cells (trans-costimulation). Eur. J. Immunol. *24*, 859–866.

Green, E., and Flavell, R. (1999). TRANCE-RANK, a new signal pathway involved in lymphocyte development and T cell activation. J. Exp. Med. *189*, 1017–1020.

Green, E.A., Wong, F.S., Eshima, K., Mora, C., and Flavell, R.A. (2000). Neonatal tumor necrosis factor alpha promotes diabetes in nonobese diabetic mice by CD154-independent antigen presentation to CD8(+) T cells. J. Exp. Med. *191*, 225–238.

Grewal, I., and Flavell, R. (1998). CD40 and CD154 in cell-mediated immunity. Annu. Rev. Immunol. *16*, 111–135.

Grewal, I., Xu, J., and Flavell, R. (1995). Impairment of antigen-specific T-cell priming in mice lacking CD40 ligand. Nature *378*, 617–620.

Grewal, I.S., Foellmer, H.G., Grewal, K.D., Xu, J., Hardardottir, F., Baron, J.L., Janeway, C.A., Jr., and Flavell, R.A. (1996). Requirement for CD40 ligand in costimulation induction, T-cell activation, and experimental allergic encephalomyelitis. Science *273*, 1864–1868.

Guerder, S., Picarella, D., Linsley, P., and Flavell, R. (1994). Costimulator B7-1 confers antigen-presenting-cell function to parenchymal tissue and in conjunction with tumor necrosis factor alpha leads to autoimmunity in transgenic mice. Proc. Natl. Acad. Sci. USA *91*, 5138–5142.

Gurunathan, S., Irvine, K., Wu, C., Cohen, J., Thomas, E., Prussin, C., Restifo, N., and Seder, R. (1998). CD40 ligand/trimer DNA enhances both humoral and cellular immune responses and induces protective immunity to infectious and tumor challenge. J. Immunol. *161*, 4563–4571.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., and Akira, S. (2000). A Toll-like receptor recognizes bacterial DNA. Nature *408*, 740–745.

Hermann, P., Van-Kooten, C., Gaillard, C., Banchereau, J., and Blanchard, D. (1995). CD40 ligand-positive CD8+ T cell clones allow B cell growth and differentiation. Eur. J. Immunol. *25*, 2972–2977.

Hoglund, P., Mintern, J., Waltzinger, C., Heath, W., Benoist, C., and Mathis, D. (1999). Initiation of autoimmune diabetes by developmentally regulated presentation of islet cell antigens in the pancreatic lymph nodes. J. Exp. Med. *189*, 331–339.

Kane, L., Lin, J., and Weiss, A. (2000). Signal transduction by the TCR for antigen. Curr. Opin. Immunol. *12*, 242–249.

Katz, J., Benoist, C., and Mathis, D. (1993). MHC class I molecules are required for the generation of insulitis in non-obese diabetic mice. Eur. J. Immunol. *23*, 3358–3360.

Kishimoto, K., Dong, V.M., Issazadeh, S., Fedoseyeva, E.V., Waaga, A.M., Yamada, A., Sho, M., Benichou, G., Auchincloss, H., Jr., Grusby, M.J., et al. (2000). The role of CD154-CD40 versus CD28-B7 costimulatory pathways in regulating allogeneic Th1 and Th2 responses in vivo. J. Clin. Invest. *106*, 63–72.

Koch, F., Stanzl, U., Jennewein, P., Janke, K., Heufler, C., Kämpgen, E., Romani, N., and Schuler, G. (1996). High level IL-12 production by murine dendritic cells: upregulation via MHC class II and CD40 molecules and downregulation by IL-4 and IL-10. J. Exp. Med. *184*, 741–746.

Koppenhoefer, U., Brenner, B., Lang, F., and Gulbins, E. (1997). The CD40-ligand stimulates T lynphocytes via the neural sphingomyelinase: a novel function of the CD40-ligand as a signaling molecule. FEBS Lett. *414*, 444–448.

Lefrancois, L., Olson, S., and Masopust, D. (1999). A critical role for CD40-CD40 ligand interactions in amplification of the mucosal CD8 T cell response. J. Exp. Med. *190*, 1275–1284.

Lu, Z., Yuan, L., Zhou, X., Sotomayor, E., Levitsky, H.I., and Pardoll, D.M. (2000). CD40-independent pathways of T cell help for priming of CD8 CTL. J. Exp. Med. *191*, 541–550.

Lutz, M.B., Kukutsch, N., Ogilvie, A.L., Rossner, S., Koch, F., Romani, N., and Schuler, G. (1999). An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J. Immunol. Methods *223*, 77–92.

Mandelbrot, D.A., Kishimoto, K., Auchincloss, H., Jr., Sharpe, A., and Sayegh, M. (2001). Rejection of mouse cardiac allografts by costimulation in *trans*. J. Immunol. *167*, 1174–1178.

Moodycliffe, A.M., Shreedhar, V., Ullrich, S.E., Walterscheid, J., Bucana, C., Kripke, M.L., and Flores-Romo, L. (2000). CD40-CD40 ligand interactions in vivo regulate migration of antigen-bearing dendritic cells from the skin to draining lymph nodes. J. Exp. Med. *191*, 2011–2020.

Moritani, M., Yoshimoto, K., Li, S., Kondo, M., Iwahana, H., Yamaoka, T., Sano, T., Nakano, N., Kikutani, H., and Itakura, M. (1996). Prevention of adoptively transferred diabetes in NOD mice with IL-10-transduced islet-specific Th1 lymphocytes. A gene therapy model for autoimmune diabetes. J. Clin. Invest. *98*, 1851–1859.

Nagata, M., Santamaria, P., Kawamura, T., Utsugi, T., and Yoon, J.-W. (1994). Evidence for the role of CD8+ cytotoxic T cells in the destruction of pancreatic beta cells in NOD mice. J. Immunol. *152*, 2042–2050.

Newell, K., He, G., Guo, Z., Kim, O., Szot, G.L., Rulifson, I., Zhou, P., Hart, J., Thistlethwaite, J.R., and Bluestone, J.A. (1999). Cutting edge: blockade of the CD28/B7 costimulatory pathway inhibits intestinal allograft rejection mediated by CD4+ but not CD8+ T-cells. J. Immunol. *163*, 2358–2362.

Peng, X., Kasran, A., Warmerdam, P.A., de Boer, M., and Ceuppens, J.L. (1996). Accessory signaling by CD40 for T cell activation: induction of Th1 and Th2 cytokines and synergy with interleukin-12 for interferon-gamma production. Eur. J. Immunol. *26*, 1621–1627.

Renshaw, B.R., Fanslow, W.C., III, Armitage, R.J., Campbell, K.A., Liggitt, D., Wright, B., Davison, B.L., and Maliszewski, C.R. (1994). Humoral immune responses in CD40 ligand-deficient mice. J. Exp. Med. *180*, 1889–1900.

Ridge, J., Rosa, F.D., and Matzinger, P. (1998). A conditioned dendritic cell can be a temporal bridge between a CD4+ T helper and a T-killer cell. Nature *393*, 474–478.

Roy, M., Waldschmidt, T., Aruffo, A., Ledbetter, J., and Noelle, R. (1993). The regulation of the expression of gp39, the CD4 ligand, on normal and cloned CD4+ T cells. J. Immunol. *151*, 2497–2510.

Ruedl, C., Kopf, M., and Bachmann, M.F. (1999). CD8(+) T cells mediate CD40-independent maturation of dendritic cells in vivo. J. Exp. Med. *189*, 1875–1884.

Sad, S., Krishnan, L., Bleakley, R., Kagi, D., Hengartner, H., and Mossman, T. (1997). Cytotoxicity and weak CD40 ligand expression of CD8+ type 2 cytotoxic T-cells restrict their potential B cell helper activity. Eur. J. Immunol. *27*, 914–922.

Santamaria, P., Utsugi, T., Park, B., Averill, N., Kawazu, S., and Yoon, J. (1995). Beta cell cytotoxic CD8+ T cells from non-obese diabetic mice use highly homologous T cell receptor alpha chain CDR3 sequences. J. Immunol. *154*, 2494–2503.

Schmidt, D., Verdaguer, J., Averill, N., and Santamaria, P. (1997). A mechanism for the major histocompatibility complex-linked resistance to autoimmunity. J. Exp. Med. *186*, 1059–1075.

Schoenberger, S.P., Toes, R.E., van der Voort, E.I., Offringa, R., and Melief, C.J. (1998). T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. Nature *393*, 480–483.

Schuurhuis, D.H., Laban, S., Toes, R.E., Ricciardi-Castagnoli, P., Kleijmeer, M.J., van der Voort, E.I., Rea, D., Offringa, R., Geuze, H.J., Melief, C.J., and Ossendorp, F. (2000). Immature dendritic cells acquire CD8+ CTL priming capacity upon activation by T helper cell-independent or -dependent stimuli. J. Exp. Med. *192*, 145–150.

Serreze, D., Leiter, E., Christianson, G., Greiner, D., and Roopenian, D. (1994). Major histocompatibility complex class I-deficient NOD.$\beta 1m^{null}$ mice are diabetes and insulitis resistant. Diabetes *43*, 505–508.

Shinde, S., Wu, Y., Guo, Y., Niu, Q., Xu, J., Grewal, I., Flavell, R., and Liu, Y. (1996). CD40L is important for induction of, but not response to, costimulatory activity. ICAM-1 as the second costimulatory molecule rapidly up-regulated by CD40L. J. Immunol. *157*, 2764–2768.

Stephens, L., and Kay, T.W.H. (1995). Pancreatic expression of B7 costimulatory molecules in the non-obese diabetic mouse. Int. Immunol. *7*, 1885–1895.

Stout, R., and Suttles, J. (1996). The many roles of CD40:CD40L interactions in cell-mediated imflammatory responses. Immunol. Today *17*, 487–491.

Thivolet, C., Bendelac, A., Bedossa, P., Bach, J.-F., and Carnaud, C. (1991). CD8+ T cell homing to the pancreas in the nonobese diabetic mouse is CD4+ T cell-dependent. J. Immunol. *146*, 85–88.

Tisch, R., and McDevitt, H. (1996). Insulin-dependent diabetes mellitus. Cell *85*, 291–297.

Trambley, J., Bingaman, A.W., Lin, A., Elwood, E.T., Waitze, S.Y., Ha, J., Durham, M.M., Corbascio, M., Cowan, S.R., Pearson, T.C., and Larsen, C.P. (1999). Asialo GM1(+) and CD8(+) T cells play a critical role in costimulation blockade-resistant allograft rejection. J. Clin. Invest. *104*, 1715–1722.

van Essen, D., Kikutani, H., and Gray, D. (1995). CD40 ligand-transduced co-stimulation of T-cells in the development of helper function. Nature *378*, 620–623.

Verdaguer, J., Yoon, J.-W., Anderson, B., Averill, N., Utsugi, T., Park, B.-J., and Santamaria, P. (1996). Acceleration of spontaneous diabetes in TCRβ-transgenic NOD mice by beta cell-cytotoxic CD8+ T cells expressing identical endogenous TCRα chains. J. Immunol. *157*, 4726–4735.

Verdaguer, J., Schmidt, D., Amrani, A., Anderson, B., Averill, N., and Santamaria, P. (1997). Spontaneous autoimmune diabetes in monoclonal T cell nonobese diabetic mice. J. Exp. Med. *186*, 1663–1676.

Wang, B., Gonzalez, A., Benoist, C., and Mathis, D. (1996). The role of CD8+ T-cells in initiation of insulin-dependent diabetes mellitus. Eur. J. Immunol. *26*, 1762–1769.

Whitmire, J., and Ahmed, R. (2000). Costimulation in anti-viral immunity: differential requirements for CD4+ and CD8+ T cell responses. Curr. Opin. Immunol. *12*, 448–455.

Wicker, L.S., Leiter, E.H., Todd, J.A., Renjilian, R.J., Peterson, E., Fischer, P.A., Podolin, P.L., Zijlstra, M., Jaenisch, R., and Peterson, L.B. (1994). β2-microglobulin-deficient NOD mice do not develop insulitis or diabetes. Diabetes *43*, 500–504.

Wong, F.S., Karttunen, J., Dumont, C., Wen, L., Visintin, I., Pilip, I.M., Shastri, N., Pamer, E.G., and Janeway, C.A., Jr. (1999). Identification of an MHC class I-restricted autoantigen in type 1 diabetes by screening an organ-specific cDNA library. Nat. Med. *9*, 1026–1031.

Yang, Y., and Wilson, J. (1996). CD40 ligand-dependent T-cell activation: requirement of B7-CD28 signalling through CD40. Science *273*, 1862–1865.